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# Multiplex Reverse Transcription-PCR for Surveillance of Influenza A and B Viruses in England and Wales in 1995 and 1996

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**Multiple-target (multiplex) reverse transcription-PCR (RT-PCR) for detection, typing, and subtyping of the hemagglutinin gene of influenza type A (H3N2 and H1N1) and type B viruses was developed and applied prospectively to virological surveillance of influenza in England in the 1995–1996 winter season. During this season both influenza A H3N2 and H1N1 viruses were circulating, although at different times. Six hundred nineteen combined nose and throat swabs taken by general practitioners in sentinel practices from individuals presenting with "influenzalike illness" were analyzed by culture, multiplex RT-PCR, and immunofluorescence. Of the 619 samples, 246 (39.7%) were positive by multiplex RT-PCR compared with 200 (32.3%) which yielded influenza viruses on culture. There was 100% correlation between multiplex RT-PCR typing and subtyping and the influenza types and subtypes obtained from culture. There was also excellent correlation between the temporal detection of influenza A H3N2 and H1N1 viruses by multiplex RT-PCR and by culture. During the peak weeks of influenza virus activity, a total of 259 specimens were received, of which 101 (38.9%) yielded influenza viruses on culture while 149 (57.5%) were positive in multiplex RT-PCR, providing an increase in detection of influenza viruses of approximately 20%. The increased detection of influenza virus occurred in all the age groups sampled. Samples which were positive by multiplex RT-PCR but negative by culture were not detected significantly earlier or later in the winter of 1995–1996 but were detected during the peak weeks of clinical influenza virus activity. Multiplex RT-PCR was successfully used in surveillance of influenza to provide accurate, sensitive diagnosis directly on clinical specimens sent through the post.**

There are two major types of influenza virus, types A and B, which are responsible for disease in humans. Disease resulting from influenza virus infection ranges from mild respiratory illness to fatal pneumonia, and influenza is a significant cause of morbidity and mortality throughout the world. Excess mortality associated with influenza epidemics depends on the novelty of the virus strain, but even in a year of a comparatively minor epidemic, such as 1993, excess mortality in England and Wales was estimated at 13,000, and in 1989, a year of a more serious epidemic, it was estimated at 25,000 (13). The elderly and individuals with underlying cardiorespiratory disease are most at risk from the complications of influenza.

Influenza viruses are segmented, negative-stranded RNA viruses. Both influenza A and B viruses display a progressive antigenic change known as antigenic drift, and additionally, influenza A virus occasionally undergoes a more radical antigenic change known as antigenic shift. Influenza vaccines in common use comprise three virus subunits which are updated annually to take account of the antigenic drift in natural isolates. In recent years, intensive worldwide influenza surveillance has provided information which allows formulation of a subunit vaccine that is a good antigenic match for the circulating epidemic viruses (5, 25). In many countries, sentinel networks for the collection of both epidemiological data and samples for virological examination have been established (15). In England and Wales, the Royal College of General Practitioners (RCGP) has established a network of 91 sentinel practices from which clinical data are collected on the incidence and severity of respiratory illness throughout the year. A small number of these practices take respiratory samples for virological analysis from individuals presenting with "influenza and influenzalike illness," providing combined virological and clinical surveillance (14). The early identification of circulating strains in each winter season, which is made possible through such surveillance schemes, provides important information to public health authorities and enables appropriate vaccination or prophylactic treatment of high-risk groups.

Virological analysis usually takes the form of isolation and typing of virus from nose and throat swabs with tissue culture or embryonated chicken eggs and/or of antigen detection by immunofluorescence (IF) or enzyme-linked immunosorbent assay. Another approach to the rapid detection and identification of influenza viruses is the application of reverse transcription-PCR (RT-PCR) for influenza virus. Influenza A and B virus genomes have been detected in clinical specimens, including nasopharyngeal aspirates, nose and throat swabs, and bronchoalveolar lavage fluid (8, 27, 28). However, there has been only limited evaluation of the application of RT-PCR for influenza viruses compared to the conventional methods of virus isolation and IF in community-based surveillance. We have developed a multiplex RT-PCR for the detection, typing, and subtyping of influenza viruses in combined nose and throat swabs taken for surveillance of influenza in England and Wales.

Multiplex RT-PCR involves the simultaneous amplification of more than one target sequence in a single reaction tube, using more than one primer pair. The advantage of developing a multiplex RT-PCR for influenza A H1N1 and H3N2 and influenza B viruses is that it combines the sensitivity and rapidity of PCR but avoids the need to test clinical specimens separately for each virus subtype. We describe the development, optimization, and evaluation of a multiplex RT-PCR for typing and subtyping influenza viruses in the postal surveillance samples collected through the RCGP surveillance

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scheme for influenza during the 1995–1996 winter season in England and Wales and compare the results to those of virus isolation and IF.

## MATERIALS AND METHODS

**Epidemiology.** Data on the rates of influenza and influenza like illness were collected by 91 RCGP sentinel practices well distributed throughout England and Wales and covering a population of 691,000. All new episodes of illness are recorded, indexed, and reported weekly to the Birmingham Research Unit, where they are analyzed to provide incidence data, presented as new episodes per 100,000 individuals. A subset of practices also takes part in virological monitoring as previously described.

**Clinical specimens.** Nose and throat swabs taken from individuals presenting with influenza and influenzalike illness were combined in 3 ml of virus transport medium (VTM) by physicians participating in the RCGP surveillance scheme. The samples were sent in the post to the National Influenza Laboratory for virological analysis. For RT-PCR analysis, an aliquot was removed on receipt of the specimen, prior to any manipulation, and stored at −70°C until it was tested.

**Virus isolation and growth.** Specimens were vortexed for 10 s to remove cells which remained adherent to swabs, and 100 μl of each specimen was inoculated onto confluent Madin-Darby canine kidney (MDCK) or primary rhesus monkey kidney (RMK) cells. The cells were maintained postinoculation in minimal essential medium (Biowhittaker) supplemented with penicillin and streptomycin. In addition, 1.25 μg of tolylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin/ml (Worthington Laboratories) was added to the medium for MDCK cells. Both MDCK and RMK cells were incubated with rolling at both 33 and 37°C. The medium was tested at days 7 and 14 for hemagglutination activity with turkey erythrocytes, and prior to discard, a hemadsorption test was performed with guinea pig erythrocytes (9). Cells which showed hemadsorption but no hemagglutination were further tested by IF for parainfluenza 1 to 3 and mumps viruses with commercial monoclonal antibodies (catalog no. MAB819 and MAB846; Chemicon).

**Immunofluorescence.** Clinical specimens were centrifuged at 1,000 × *g* in an MSE Mistral 3000 benchtop centrifuge for 10 min. The resulting cellular pellet was washed three times in ice-cold phosphate-buffered saline (PBS) (pH 7.4) and resuspended in PBS. An aliquot of cell suspension was air dried onto glass slides and fixed for 10 min in acetone at −20°C, and indirect immunofluorescence for influenza A and B viruses was performed with monoclonal antibodies (catalog no. 5017 and 5018; Chemicon).

**Virus typing.** Tissue culture fluids from inoculated cells were tested regularly for hemagglutination activity, and those with such activity were typed in hemagglutination inhibition (HI) tests with ferret antisera to influenza A H1N1 and H3N2 and influenza B prototype viruses, as described previously (2). All ferret antisera were treated with a receptor-destroying enzyme from *Vibrio cholerae*. All HI tests were carried out with 8 hemagglutinating units of virus and 0.5% (vol/vol) turkey erythrocytes.

**Nucleic acid extraction and cDNA synthesis.** RNA was extracted from a 100-μl sample with guanidinium thiocyanate (1). Egg fluids, tissue culture material, clinical specimens, and VTM were treated in the same manner. Viral RNA was eluted in 30 μl of water, and for cDNA synthesis, 22.2 μl of RNA was added to an RT reaction mixture (17.8 μl) containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 7.5 mM $MgCl_2$, 1.5 mM (each) deoxynucleoside triphosphate, 25 ng of random primer $(pdN)_6$ (Pharmacia), 1.6 U of RNasin (Promega), and 200 U of Moloney murine leukemia virus reverse transcriptase (Gibco BRL). The reaction mixture was incubated at room temperature for 10 min and then at 37°C for 45 min. Samples were then heated to 100°C for 5 min and cooled on ice. All clinical samples were extracted in batches of 20, along with positive controls for each influenza type and subtype virus and a negative control of VTM. Control samples were included at the end of each batch.

Influenza virion RNA was prepared from allantoic fluid harvested from 12-day-old embryonated eggs infected at 10 days with influenza virus. Allantoic fluid was clarified by centrifugation at 1,000 × *g* in an MSE Mistral 6000 centrifuge, and polyethylene glycol 6000 was added to a final concentration of 6% (wt/vol). Allantoic fluid was then centrifuged for 16 h at 4°C at 1,000 × *g*. The resultant pellet was resuspended in 10 mM Tris-HCl, pH 8.0, and centrifuged for 90 min on a continuous 40 to 15% sucrose gradient in a Beckmann SW27 rotor at 23,000 rpm at 4°C. The virus band was harvested and pelleted in PBS prior to RNA extraction. The pellet was dissolved in ammonium acetate, pH 4.5, and extracted as described above. The RNA concentration was assessed by spectrophotometry at 260 and 280 nm.

**PCR.** For detection of influenza A H1 and H3 or influenza B viruses with single primer sets (uniplex RT-PCR) or by multiplex RT-PCR, we used previously published nested primer sets (28). The properties of the primers were analyzed with Oligo primer analysis software (version 5.0; National Biosciences Inc.). Each primer set was used at 5 pmol in the first amplification and at 25 pmol in the second amplification. Biochemical optimization of the amplification conditions for each set of nested primers was performed under a range of $MgCl_2$, salt, and pH conditions (Opti-Prime PCR optimization kit; Stratagene). Cycling conditions were as previously described (10), except that 35 cycles were used in both the first and second rounds of amplification. Amplicons were visualized by ethidium bromide staining following electrophoresis on 1.2% agarose gels.

TABLE 1. Properties of influenza PCR primers

| Primer[a] | 5′ position | Product size (bp) | $T_m$[b] (°C) | GC content (%) | Optimal annealing temp[c] (°C) | Maximal annealing temp (°C) |
|---|---|---|---|---|---|---|
| Primary amplification | | | | | | |
| AH1 A | 76 | | 55 | 40 | | |
| AH1 FII | 1090 | 1,015 | 72 | 50 | 52 | 63 |
| AH3 A | 174N | | 55 | 40 | | |
| AH3 DII | 1056 | 883 | 62 | 50 | 52 | 62 |
| BHA A | 154N | | 56 | 50 | | |
| BHA DII | 1053 | 900 | 65 | 45 | 53 | 64 |
| Secondary amplification | | | | | | |
| AH1 B | 96 | | 63 | 45 | | |
| AH1 EII | 1039 | 944 | 55 | 45 | 52 | 62 |
| AH3 B | 348 | | 63 | 45 | | |
| AH3 CII | 938 | 591 | 65 | 50 | 54 | 69 |
| BHA B | 196 | | 61 | 35 | | |
| BHA CII | 962 | 767 | 68 | 55 | 54 | 67 |

[a] Primers are named according to Zhang and Evans (28). Primers are complementary to the virion RNA, except where II indicates primers complementary to the cDNA.

[b] $T_m$, melting temperature of an oligonucleotide complex calculated by the nearest-neighbor method.

[c] The annealing temperature that gives the highest product yield when no false priming and primer dimerization occurs (calculated by Oligo version 5.0 software).

**Infectivity assays.** Infectivity assays were performed as previously described (22). Confluent MDCK cells were washed with PBS and incubated for 1 h at room temperature with virus inoculum diluted in minimal essential medium. The inoculum was then removed, and the cells were overlaid with medium containing 1% indubiose, nonessential amino acids, and 3 μg of tolylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin/ml and incubated at 37°C in 5% $CO_2$ for 48 h. The cells were fixed with 5% (vol/vol) glutaraldehyde and stained with 2% (vol/vol) carbolfuchsin.

## RESULTS

**Optimization of multiplex RT-PCR.** The oligonucleotide primers selected for amplification of the hemagglutinin (HA) gene of influenza A H1N1 and H3N2 and influenza B viruses were analyzed to ensure that they not only met the essential criteria for optimal PCR primers (6) but also could be used together in a multiplex reaction under similar conditions for amplification and produce products which could clearly be differentiated on the basis of size. All of the primers chosen were 20-mers and had a GC content of less than or equal to 55% (Table 1). The theoretical optimal and maximal annealing temperatures of the primer sets differed by less than or equal to 5°C. Annealing temperatures of 50 and 60°C in the first and second rounds of amplification, respectively, were determined to give maximum product yields and specificity (data not shown). Primers were also analyzed for the formation of dimers either within or between pairs. Although one primer, BHA B, could theoretically form a stable primer dimer (as calculated with the duplex option in Oligo version 5.0), the theoretical dimerization did not involve the 3′ termini and no reduction in the sensitivity of detection of influenza B virus either by a single primer set or in multiplex RT-PCR was observed.

Biochemical optimization of the amplification conditions for each primer set was performed, and final conditions of 1.5 mM $MgCl_2$, 25 mM KCl, and pH 8.8 were found to be optimal for

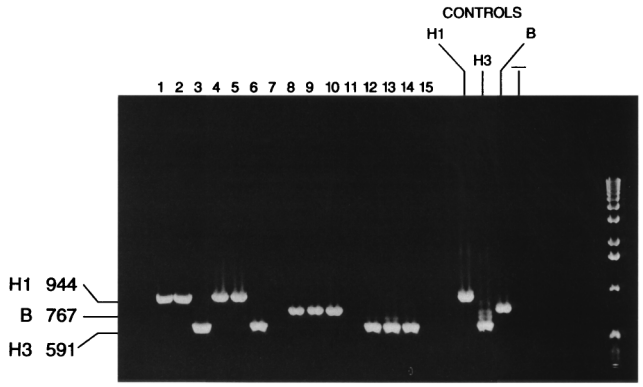


FIG. 1. Typing and subtyping of influenza viruses by multiplex RT-PCR. Combined nose and throat swabs (lanes 1 to 15) and control egg fluids (A/Texas/36/91 [H1N1], A/Beijing/32/92 [H3N2], and B/Panama/45/90 [B]) (H1, H3, and B, respectively) and uninfected VTM (−) (negative control) were assayed by multiplex RT-PCR. Amplicons were analyzed by electrophoresis on a 1.2% agarose gel stained with ethidium bromide. Molecular size markers are shown in the far right lane.

maximum yield of the specific product for each nested primer set (data not shown). The influenza A H1N1 and H3N2 and influenza B virus primer sets, when used together in the multiplex reaction, amplified only specific products of the expected sizes, 944, 591, and 767 bp, respectively, which could be easily distinguished by agarose gel electrophoresis (Fig. 1).

**Sensitivity of multiplex RT-PCR.** The sensitivity of detection of influenza virus with nested primer sets used individually and in a multiplex reaction was determined by two methods. Firstly, a dilution series of freshly harvested egg- or tissue culture-grown virus was made in VTM. From each dilution, nucleic acid was extracted for cDNA synthesis and an equivalent aliquot was taken for infectivity assays, which were set up on the same day. cDNA synthesis was followed by PCR with primer sets used individually and in a multiplex reaction. Thus, the end point of detection of infectious virus could be directly compared with the end point of detection of viral RNA by RT-PCR. RT-PCR detected 1 to 5 PFU of influenza virus with each of the three primer sets for influenza A H1N1 and H3N2 and influenza B viruses. The end point of detection for each virus type and subtype remained the same in the presence of multiple primer sets, with no apparent decrease in the yield of the PCR product. Secondly, influenza A H3N2 and H1N1 virion RNA was purified by sucrose density gradient centrifugation and quantified by ultraviolet spectrophotometry at 260 and 280 nm. Serial dilutions of purified virion RNA were prepared and subjected to RT-PCR. Dilutions containing less than 1 pg of influenza A virion RNA (equivalent to 40 genome copies) were reliably detected with the influenza A H1N1 and H3N2 virus primer sets. There was no loss of sensitivity when multiple primer sets were used together, compared to the same primer sets used individually, and no apparent decrease in the yield of the PCR product (Fig. 2).

**Surveillance specimens.** Between 1 October 1995 and 30 April 1996, a total of 619 combined nose and throat swab specimens were collected by general practitioners participating in the RCGP surveillance scheme from individuals presenting with influenza and influenzalike illness. These were sent by post to the National Influenza Laboratory. All of the specimens were analyzed for influenza virus by cell culture and multiplex RT-PCR. Additionally, all 433 of the specimens which were collected between 1 October 1995 and 31 January 1996 (week 40 of 1995 to week 4 of 1996) were also analyzed directly on receipt by IF for influenza A and B viruses. The majority (50.3%) of clinical samples were obtained from individuals under 16 years of age, although all ages were represented in this study (Table 2).

The peak of specimen collection occurred in weeks 46 to 50 of 1995 and correlated well with the peak of clinical influenza activity measured by the RCGP consultation index per 100,000 population (Fig. 3). The peak of virus isolation also occurred between weeks 46 and 50 of 1995 (Fig. 3), as did the peak of detection of influenza virus by immunofluorescence (data not shown). A total of 200 of 619 samples (32.3%) yielded influenza viruses by culture. Of the 200 influenza viruses isolated, 147 (73.5%) were influenza A H3N2 subtype and 51 (26.3%) were influenza A H1N1. Only two influenza B virus isolates were obtained from this surveillance scheme during the winter of 1995–1996. The influenza A H3N2 viruses were all obtained between 1 October 1995 (week 40 of 1995) and 28 February 1996 (week 7/96). All but three of the influenza A H1N1 viruses were obtained after 31 January 1996 (week 4 of 1996). Thus, although both influenza A H1N1 and H3N2 viruses cocirculated during the winter season of 1995–1996, there was temporal separation between the detection of the two virus types in the community. The peak weeks for detection of influenza A H3N2 virus occurred during weeks 46 to 50 of 1995, whereas the peak weeks for detection of influenza A H1N1 virus occurred during weeks 7 to 12 of 1996 (Fig. 3). Circulation of influenza A H1N1 virus in the community did not appear to have as great a correlation with the RCGP consultation index as did circulation of influenza A H3N2 virus, although during the peak weeks of circulation of influenza A H3N2 and influenza A H1N1 viruses, equivalent proportions of swabs yielded viruses (Fig. 3).

**Multiplex RT-PCR.** Influenza A (H1N1 or H3N2) or B virus was detected in a total of 246 of 619 samples (39.7%) by multiplex RT-PCR. A total of 72 of 619 samples (11.6%) were

TABLE 2. Age range of patients providing surveillance specimens

| Age range (yr) | No. of specimens (%) | Virus detected by: | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Culture | | | | PCR (%) | Culture and PCR (%) | PCR only (%) | Culture only (%) |
| | | Total (%) | H1N1 | H3N2 | B | | | | |
| 0–5 | 197 (30.8) | 61 (30.9) | 10 | 51 | 0 | 74 (37.5) | 55 (27.9) | 19 (9.6) | 6 (3.0) |
| 5–16 | 121 (19.5) | 59 (48.7) | 28 | 31 | 0 | 69 (57.0) | 52 (42) | 18 (14.8) | 7 (6.2) |
| 16–35 | 139 (22.5) | 41 (29.4) | 7 | 34 | 0 | 53 (38.0) | 37 (26.6) | 17 (12.2) | 4 (3.0) |
| 35–65 | 110 (17.7) | 24 (21.8) | 3 | 21 | 0 | 32 (29.0) | 19 (17.2) | 13 (11.8) | 5 (4.5) |
| >65 | 38 (6.1) | 10 (26.3) | 0 | 10 | 0 | 13 (34.2) | 9 (23.6) | 4 (10.5) | 1 (2.6) |
| Unknown | 14 (1.5) | 5 (35.7) | 3 | 0 | 2 | 5 (35.7) | 4 (28.5) | 1 (7.1) | 1 (7.1) |
| Total | 619 | 200 | 51 | 147 | 2 | 246 | 176 | 72 | 24 |

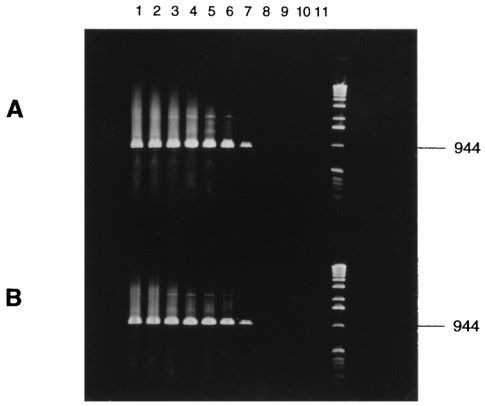


FIG. 2. Determination of assay sensitivity. Serial dilutions of purified influenza A H1N1 (A/Taiwan/1/86) virion RNA were prepared in VTM. Each dilution was subjected to RT-PCR with the AH1 primer set alone (A) and with the AH1, AH3, and B primer sets in a multiplex reaction (B). Lanes: 1, 0.3 μg; 2, 30 ng; 3, 3.0 ng; 4, 0.3 ng; 5, 30 pg; 6, 3.0 pg; 7, 0.3 pg; 8, 30 fg; 9, 3.0 fg; 10, 0.3 fg; 11, blank. Molecular size markers are in the far right lane. The position of H1N1 (944) is marked on the right.

detected by multiplex RT-PCR but were not detected by tissue culture (Table 2). The detection of influenza virus by multiplex RT-PCR exceeded the detection by virus culture in every age group studied (Table 2) by approximately equivalent numbers. There was no age group in which detection of influenza virus by multiplex RT-PCR, rather than by virus isolation, appeared particularly favorable (Table 2). Of 200 specimens in which influenza virus was detected by virus culture, 176 (88%) also had virus detected by multiplex RT-PCR, and there was 100% correlation between the typing and subtyping assigned by multiplex RT-PCR and that assigned by traditional HI typing. All the virus isolates obtained from samples which were negative by RT-PCR were amplified in the multiplex PCR to ensure that failure to identify virus nucleic acid in the original samples was not due to primer mismatch (data not shown).

The peak weeks for detection of influenza virus by multiplex RT-PCR correlated precisely with the peak weeks for detection of influenza virus by culture (Fig. 3). In the peak weeks of clinical activity (weeks 46 to 50 of 1995), the detection of influenza A H3N2 virus by multiplex RT-PCR exceeded the detection of influenza A H3N2 virus by culture by up to two times (Fig. 3). Influenza virus was not detected significantly earlier or later in the influenza season by multiplex RT-PCR, but the onset of both influenza A H3N2 and H1N1 virus activity was detected by multiplex RT-PCR simultaneously with their detection by tissue culture (Fig. 3).

**Virus culture.** One hundred forty-seven isolates of influenza A H3N2 virus were detected by culture, of which 131 (89%) were also detected by multiplex RT-PCR. Fifty-one isolates of influenza A H1N1 virus were isolated in culture, and 44 isolates (87%) were detected by multiplex RT-PCR (Table 2). Two isolates of influenza B virus were identified in cell culture, and one was also detected by multiplex RT-PCR. The peak of virus detection by culture occurred for patients between 5 and 16 years of age, with 49% of the samples from this age group yielding an influenza virus (Table 2). Other age groups sampled had lower, but similar, rates of detection of viruses, ranging from 20 to 30% (Table 2). Twenty-four samples (3.8%) yielded influenza virus on culture but were negative by multiplex RT-PCR. The samples which were positive by culture but negative by multiplex RT-PCR were distributed proportionately throughout all the age groups. All of the virus isolates obtained from these samples were amplified in multiplex RT-PCR to ensure that failure to identify virus was not due to primer mismatch, as described above.

**Immunofluorescence.** Of the total 619 samples, 433 (69.9%) were also tested by IF for influenza A and B viruses. These samples were taken between 1 October 1995 and 31 January 1996, a period corresponding to peak influenza activity in England and Wales (Fig. 3). Of the 433 samples, 123 (28.4%) were positive in IF for influenza A virus and 144 (33%) yielded influenza viruses by culture, of which 140 were H3N2, 3 were H1N1, and 1 was influenza B.

Of the 123 samples which were positive in IF, 88 (70.7%) were also positive by multiplex RT-PCR, compared with only 77 samples (62.6%) which were also positive by culture (Table 3). Thus, multiplex RT-PCR confirmed more IF-positive samples than did virus culture.

Overall, only 66 of the 433 samples (15.2%) were positive by

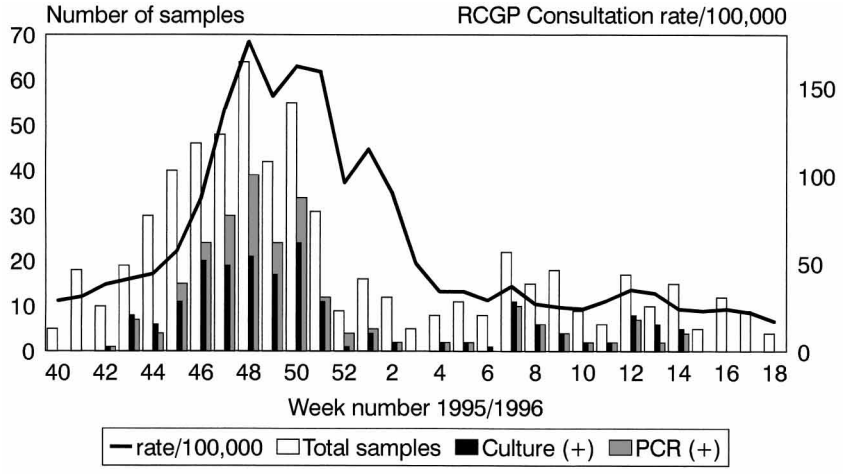


FIG. 3. The total number of combined nose and throat swabs taken by physicians participating in the RCGP surveillance scheme and the number of virus isolates and multiplex RT-PCR-positive samples derived from these swabs are shown in the histogram. The solid line represents the RCGP consultation rate per 100,000 population during the winter of 1995–1996, which is derived from the RCGP weekly returns for influenza and influenzalike illness. Culture (+) and PCR (+), number of samples in which culture and RT-PCR, respectively, detected influenza virus.

TABLE 3. Comparison of IF, culture, and multiplex RT-PCR for detection of influenza A and B viruses for surveillance samples from October 1995 to January 1996 ($n$ = 433)

| Assay (result) | No. of samples | IF (positive) (%) | Multiplex RT-PCR (positive) (%) | Culture (positive) (%) |
|---|---|---|---|---|
| IF (positive) | 123 | | 88 (71.5) | 77 (62.6) |
| IF (negative) | 310 | | 101 (32.5) | 77 (24.8) |
| RT-PCR (positive) | 189 | 87 (46) | | 124 (65.6) |
| RT-PCR (negative) | 244 | 34 (13.9) | | 20 (8.1) |
| Culture (positive) | 144 | 76 (52.7) | 124 (86) | |
| Culture (negative) | 289 | 47 (16.2) | 65 (22.4) | |

all three tests compared with 28.4% for IF, 33% by culture, and 43.6% for RT-PCR alone. Although the peak of detection of IF-positive samples corresponded to the peak of virus culture and the peak of detection of RT-PCR-positive samples (data not shown), a total of 24 of 123 IF-positive samples (19.5%) were unconfirmed by either culture or multiplex RT-PCR (Table 3), indicating that either there is a substantial proportion of false-positive IF test results on these specimens, which are not optimal for influenza virus identification by IF, or that there is a subset of clinical samples containing influenza virus antigen that neither PCR nor virus culture can identify.

**Specificity of Multiplex RT-PCR.** There was 100% correlation between the virus types and subtypes assigned by multiplex RT-PCR and those assigned by antigenic typing of the 174 specimens which were detected both by multiplex RT-PCR and by culture throughout the surveillance period. This study was not designed to identify viruses other than influenza virus in the surveillance samples taken by general practitioners. Nevertheless, 12 samples yielded a variety of viruses which were identified as a result of cytopathic effects in tissue culture. This included five enteroviruses, which were untyped, four parainfluenza 2 and one parainfluenza 3 viruses, and two respiratory syncytial viruses. All of the samples yielding these viruses were negative in the multiplex RT-PCR for influenza virus.

## DISCUSSION

The results presented here indicate the feasibility of using multiplex RT-PCR for detection of RNA containing viral pathogens in surveillance work. Global surveillance of influenza is intensive and is directed towards improving the information available for decision making in the annual formulation of a subunit vaccine to be used worldwide. Few countries have sentinel surveillance networks for influenza virus in which the clinical surveillance data is linked to virological analysis, as occurs in England and Wales through the RCGP network (14, 21). This provides an ideal setting in which to study the correlation of clinical diagnosis with virological confirmation.

Traditionally, this type of influenza virus surveillance work has been based on detection of influenza virus by culture with, or without, rapid antigen detection tests such as IF or enzyme-linked immunosorbent assay. Knowledge of the antigenic properties of circulating influenza viruses remains essential for formulation of vaccines which match circulating strains, and the study of the properties of influenza virus isolates will continue to underpin the annual World Health Organization recommendations for vaccine composition. However, the quality of clinical surveillance data can be improved by timely, accurate diagnosis, which can be provided by multiplex RT-PCR directly on clinical samples. Identification of influenza viruses by traditional culture methods can take up to 14 days, and the difficulties of recovering infectious virus from clinical samples are enhanced when the samples travel in the post from the clinician to the laboratory, which takes at least 24 h and occasionally up to 5 days. Thus, developing methodology which relies on RT-PCR detection of pathogens should improve the diagnostic yield when infectious virus is likely to have been inactivated due to prolonged incubation at inappropriate temperatures.

We have used a multiplex RT-PCR strategy based on detection of the HA1 portion of the hemagglutinin gene of influenza A and B viruses, because this gene codes for the major neutralizing antigen of influenza virus, the hemagglutinin protein (HA). A sensitive multiplex RT-PCR assay was achieved by careful choice of primers and optimization of the biochemical and cycling conditions. The genetic information coding for the major antigenic sites on the HA protein is contained within the amplicons generated as a result of the PCR strategy adopted here (23) (Table 1). This allows the possibility of further analysis of the amplicon, either by sequencing or by RT-PCR restriction digestion, which increases the information about the genetic profile of the virus infecting the individual and allows very rapid identification of strains at the beginning of the influenza season (11, 12). Furthermore, comparison of the virus genome contained within clinical samples and that amplified by tissue culture is possible, and may be desirable, because of the phenomenon of host cell selection seen in influenza virus growth (20).

During the winter of 1995–1996, influenza A virus H1N1 and H3N2 subtypes were cocirculating in England and Wales, although their detection was temporally separated (Fig. 3). There was excellent correlation between the temporal detection of influenza A H3N2 and H1N1 viruses by culture and by multiplex RT-PCR. No samples containing influenza A H3N2 or H1N1 virus nucleic acid were found before the first sample which yielded influenza A H3N2 or H1N1 virus on culture at the beginning of the winter season or the beginning of the period of circulation of influenza A H1N1 virus, respectively. However, many more samples were found to be positive during the peak of the season (weeks 46 to 50 of 1995) by RT-PCR than by culture (Fig. 3). The extra samples found to be positive by multiplex RT-PCR but negative by culture were all taken during the period of highest clinical activity registered by clinicians, and extra influenza diagnoses by multiplex RT-PCR were not identified beyond the period of circulation of these two viruses. Although influenza B virus did not circulate during the period of this study, analysis of the 1996–1997 influenza season indicated that influenza B virus was detected at least as well as influenza A H3N2 virus by multiplex RT-PCR compared with virus culture (data not shown).

If the results are considered in the context of peak clinical influenza virus activity, the benefits of multiplex RT-PCR detection can be more clearly seen. During the weeks of peak influenza virus activity (weeks 46 to 50 of 1995), a total of 259 specimens were received, of which 101 (38.9%) yielded influenza viruses by culture, but 149 (57.5%) of the same set of samples indicated the presence of influenza viruses by multiplex RT-PCR, providing an increase in detection of influenza viruses of approximately 20% overall. Thus, the most marked improvement in detection was obtained during the peak period when influenza virus was circulating in the community, indicating the improved positive predictive value of multiplex RT-PCR compared with virus culture. The improvement in diagnosis of influenza virus infection by multiplex RT-PCR occurred across all the age ranges of patients sampled, and was

not restricted to any particular age group (Table 2). The correlation between IF-positive specimens and multiplex RT-PCR-positive specimens was greater than that between IF-positive specimens and culture-positive specimens, which may reflect the fact that RT-PCR is able to detect noninfectious virus, but overall the concordance among all three tests was fairly low, as only 15.2% of samples were positive in all three tests compared to the 28 to 30% that were positive in two tests.

Combined nose and throat swabs are not an optimum clinical sample for detection of influenza virus by IF because they often contain an excess of squamous epithelial cells and very few respiratory epithelial cells compared to other respiratory samples, e.g., nasopharyngeal aspirates. Even if sample types are standardized, published values for concordance between IF and culture still vary between 65 and 92% (7, 17). It is likely that at least some of the IF-positive samples which were unconfirmed either by culture or by multiplex RT-PCR represented false-positive results caused by difficulty in reading fluorescence on suboptimal samples.

There was perfect correlation (100%) between types and subtypes obtained by multiplex RT-PCR and by tissue culture isolation of virus when samples were positive by both methods. By culture, 24 of 619 samples (3.8%) yielded viruses which were not detected by RT-PCR in the initial specimens. Although the same initial volume was used for culture inoculation as for nucleic acid extraction, only half of the cDNA synthesized following nucleic acid extraction and the RT reaction was taken forward into the PCR to avoid inhibition of the *Taq* polymerase by RT reaction products. Therefore, the failure to detect a small fraction of samples which yielded virus on culture may reflect a very low-titered initial inoculum rather than primer mismatch resulting in failure of amplification, as all the virus isolates obtained from these specimens did amplify when tested in RT-PCR. A possible way of increasing the sensitivity of the multiplex RT-PCR test overall would be to increase the volume of clinical material analyzed.

Approaches to the detection of influenza viruses in clinical specimens or egg-grown material have used primers specific for the matrix genes (8) or nonstructural genes (4). Other RT-PCR assays have used two primer sets specific for the H1N1 and H3N2 hemagglutinin genes to detect influenza A virus subtypes (24) or three primer sets to distinguish influenza A virus subtypes and influenza B virus in clinical material (19, 26) or culture fluids (28). There have been very few studies which have used RT-PCR for influenza viruses in surveillance work involving community-based sampling, and those which have been performed do not show an unequivocal increase in detection by RT-PCR compared to traditional virus culture (4, 26). The reasons for this are not absolutely clear, but perhaps they may pertain to sample handling or nucleic acid extraction methods, and the results probably reflect the sensitivity of tissue culture as a means of influenza virus detection. It is possible that the data in this paper may overrepresent the relative sensitivity of RT-PCR compared to culture, because only samples sent through the post have been analyzed. However, analysis of 44 nose and throat swabs taken from outbreaks of influenza in local nursing homes in 1996 and 1997, transported to the laboratory, and inoculated within 6 h indicated that multiplex RT-PCR was indeed considerably more sensitive, as only nine specimens were positive by culture for influenza virus but 17 were positive by multiplex RT-PCR (data not shown), an increase in detection of 88%. Therefore, the increase in detection obtained by using multiplex RT-PCR is maintained and possibly increased if specimens do not have to come through the post. It is likely that there is some degradation of viral nucleic acid as well as loss of virus viability when specimens are transported in this way.

Multiplex PCR assays capable of amplifying 13 or more separate regions of chromosomal DNA and of detecting and typing several bacterial and viral pathogens have also been described (3; reviewed in reference 18). However, the use of multiplex PCR for the detection of viral pathogens with RNA genomes has been much more limited, possibly due to the difficulties of overcoming the inherent inefficiency of the RT step in RT-PCR or due to the difficulties in nucleic acid extraction when the starting material may be of poor quality. Notwithstanding these potential problems, multiplex RT-PCR can be successfully used in surveillance, as demonstrated in this work, providing excellent correlation with traditional methods of influenza virus identification and allowing sensitive, rapid detection. Furthermore, the combination of sensitive tissue culture systems and multiplex RT-PCR provides the possibility of improving the verification of clinical diagnosis of influenza and identifying cases of influenzalike illness in which influenza virus is probably not the etiological agent. Identification of the negative cases is almost as important as verification of the positive cases, because it is in these cases that other pathogens should be sought. Many respiratory viruses produce a syndrome which is difficult to distinguish from true influenza, and the burden of illness caused by other viral pathogens, e.g., respiratory syncytial virus, may be severely underestimated (16), but it could be evaluated by the development of multiplex RT-PCR for a number of respiratory viruses.

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